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Enhanced Cellulase Production by a Mutant of *Trichoderma viride*

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A mutant strain that secretes twice as much cellulase as its parent was obtained by irradiating conidia of *Trichoderma viride* QM 6a with a linear accelerator.

Simple, efficient, and economical processes for conversion of cellulose to glucose by enzymatic hydrolysis would aid in alleviation of pollution problems and in developing new food sources. The glucose thus produced could be converted to yeast or other microbial protein (1, 4, 6; T. K. Ghose and J. A. Kostick, Biotechnol. Bioeng., *in press*). Many fungi rapidly degrade cellulose, but only a few produce cellulolytic enzymes of high potency and stability. Of these, *Trichoderma viride* was found to be a convenient source of a cellulase complex (C_1 + Cx enzymes) capable of total hydrolysis of native insoluble cellulose to glucose (6). Increases in cellulase yields have been achieved by optimizing cultural conditions (6) and by addition of surfactants to the medium (7). A further increase of the enzyme yield was obtained by mutating the fungus as described below.

Conidia of the parent *T. viride* QM6a (6) were suspended in distilled water and irradiated at 20 C with high energy electrons from a 24-million electron volt, 18-kw linear accelerator (3). More than 95% of the conidia were killed by a dose of 0.05 megarads, and no survivors were found above 0.2 megarads. Survivors (0.05–0.20 megarads) were plated on desoxycholate agar (BBL). On this medium, the growth was greatly restricted and the colonies did not exceed 2 mm in diameter even after long incubation. Yet sporulation occurred, and, when transferred to potato dextrose agar, the fungus grew normally. Isolates were then grown in cellulose medium (6) and examined for cellulase production. A strain designated as QM9123 was isolated (dose, 0.05 megarads) which produced twice as much cellulase as the parent strain (Table 1). Another isolate, QM9136 (dose, 0.05 megarads), did not produce cellulase. Except for cellulase production, both strains closely resembled the parent in their growth characteristics.

TABLE 1. *Comparison of cellulase produced by Trichoderma viride QM6a and QM9123*[a]

Enzyme substrate	Measurement of activity	Activity		
		Strain		Ratio of 9123:6a
		6a	9123	
Carboxymethyl cellulose (1%)	C_x units/ml, 30 min (6)	112	440	3.9
Ball-milled cellulose (1%)	"Sweco" units/ml, 30 min (4)	9	22	2.4
Filter paper (5%)	Activity, 1 hr (6)	2.6	5.0	1.9
Alpha cellulose (5%)	Glucose (mg per ml per day) (6)	8.4	14.4	1.7
Absorbent cotton (5%)	Glucose (mg per ml per day) (6)	2.3	7.1	3.1
Soluble protein	Mg/ml (5)	0.58	1.18	2.0

[a] Cultures were grown at 28 C in shaken flasks with nutrient salts, 0.5% cellulose, 0.05% proteose peptone, and 0.1% Tween 80 (6) for 14 days. The culture filtrates were tested for cellulase activities at 50 C, *p*H 4.8, in stationary test tubes (6).

The three strains were compared for growth and enzyme production on various media (Table 2). On potato dextrose agar, the colonies grew rapidly at an equal rate and covered an entire slant or plate in 2 or 3 days at 25 C. In shaken flasks, they formed dense diffuse mycelium. Pellets were not formed. On starch and sugars, growth was rapid and the substrates were consumed in 3 days. Growth on cellulose was slower,

TABLE 2. *Enzyme production by Trichoderma viride QM6a, QM9123, and QM9136 on various substrates*[a]

Growth substrate	Culture	Total protein (mg/ml)	Soluble protein (mg/ml)	Enzyme activity of filtrates		
				Cellulose (filter-paper activity)	Starch (units/ml)	β 1 → 3-glucan (units/ml)
Cellulose (0.5%)	QM6a	0.68	0.58	2.30	3.1	5.2
	QM9123	1.25	1.18	4.95	2.3	3.2
Glucose (0.5%)	QM6a	0.32	0.08	0.10	4.0	2.7
	QM9123	0.36	0.07	0.05	4.2	3.9
	QM9136	0.40	0.07	0.00	5.9	3.5
Cellobiose (1.0%)	QM6a	0.70	0.14	0.68	1.4	1.2
	QM9123	0.84	0.20	1.38	1.2	2.7
	QM9136	0.81	0.13	0.07	1.5	4.1
Lactose (1.0%)	QM6a	0.42	0.20	0.70	2.7	2.7
	QM9123	0.58	0.30	1.17	1.7	2.1
	QM9136	0.40	0.12	0.09	5.6	3.0
Starch (0.5%)	QM6a	NT[b]	NT	0.10	3.8	3.9
	QM9123	NT	NT	0.09	5.6	2.9
	QM9136	NT	NT	0.05	6.8	4.4

[a] Cultures were grown for 14 days at 28 C in shaken flasks with nutrient salts plus 0.1% Tween 80 (6) and carbon sources as shown. Cellulose cultures also contained 0.05% proteose peptone. Culture filtrates were tested for enzymatic activities at *p*H 4.8, 50 C in stationary test tubes against cellulose as filter-paper activity (6) and starch and β1 → 3 glucan (Laminarin) as units per ml. One unit releases 1.0 mg of glucose from 0.5% substrate in 30 min. Protein was measured by the phenol reagent after precipitation with 5% trichloroacetic acid (5).

[b] Not tested.

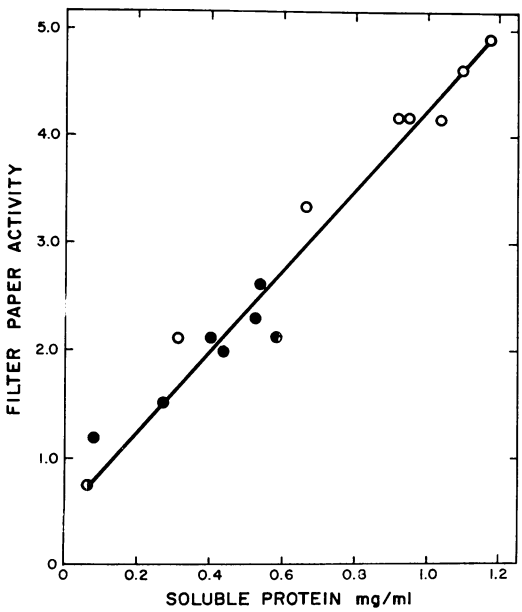

FIG. 1. *Relation between soluble protein and cellulase activity. Cultures were grown in 0.5% cellulose plus 0.05% proteose peptone and 0.1% Tween 80. Filter-paper activity and soluble protein were measured frequently over a 21-day growth period. Symbol: ●, QM6a; ○, QM9123.*

with particles of cellulose still visible microscopically after 7 days, with the exception of mutant QM9136 which did not grow on cellulose. Enzymes attacking starch and β 1 → 3 glucan were produced on all substrates by all three strains. Mutants QM6a and QM9123 produced cellulase when grown in cellobiose, lactose, and cellulose. More protein was secreted into the medium when grown in cellulose than in other substrates, and mutant QM9123 produced and secreted twice as much protein and twice as much cellulase on cellulose as did the parent QM6a. Cellulase level closely paralleled the level of soluble protein (Fig. 1). The mutant QM9123 has retained its increased activity for over 2 years with frequent subcultures on potato dextrose agar and has retained it when stored in lyophilized state.

The mutations in *T. viride* appeared to be specific for cellulase without marked effect on other carbohydrases. In contrast, a mutation of *Neurospora* induced by ultraviolet has been reported (2) that resulted in increased production of glucamylase, β-fructofuranosidase, and trehalase but did not affect production of alkaline phosphatase. Mutant QM9123 produced a greater quantity of the same cellulase produced by the parent QM6a with similar ratios of activities on different cellulose substrates. Mutant QM9136

lost all the cellulase activity and culture filtrates of this strain neither inhibited nor stimulated cellulase activity of other cellulase preparations. Despite further mutagenic treatments, this strain has never back-mutated to a cellulase-producing strain.

Cellulase from mutant QM9123 has been used to hydrolyze rapidly cellulose pulp to glucose at conversions approaching 100% and yielding glucose syrups as great as 15% (Ghose and Kostick, Biotechnol. Bioeng., *in press*). Additional improvements in this mutant would promote applications of cellulase to waste disposal and other problems.

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